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Title Page

Title: EFFECT OF VITAMIN D SUPPLEMENTATION ON FREE AND TOTAL VITAMIN D: A COMPARISON OF ASIANS
vs CAUCASIANS

Short title: VITAMIN D DOSING STUDY: ASIANS vs. CAUCASIANS

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Conflict of Interest Statement:

Dr Jaya Sujatha Gopal-Kothandapani declares no competing interests

Miss Lucy F Evans declares no competing interests

Dr Jennifer Walsh reports Speaker's honoraria from Lilly, grant funding from Alexion and

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Dr Fatma Gossiel declares no competing interests.

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Abstract:

Objectives:

It is well established that UK Asians typically have lower vitamin D levels than Caucasians. It is also known that vitamin D binding protein (DBP) is lower in some races than Caucasians. To investigate how ethnicity, skin colour and genetic variation affect the response to vitamin D (150,000 IU) administered to young Asian and Caucasian men.

Design:

Prospective, single centre clinical trial

Participants:

Sixty young men (18-25yr) of Asian (n=30) and Caucasian (n =30) origin

Measurements:

We measured serum calcium, phosphate, magnesium, alkaline phosphatase, albumin, parathyroid hormone; total 25 hydroxyvitamin D (25OHD); calculated and directly measured free 25OHD; DBP at baseline and 4 weeks; DBP genotype, skin colour (Fitzpatrick scale), dietary vitamin D and calcium intake at baseline; and urine calcium:creatinine ratio at baseline, 1 and 4 weeks.

Results

At baseline, Asians had lower serum total 25OHD (26.4 [13.7] vs 34.1 [12.3] nmol/l p=0.0272) and DBP (6.7 [3.4] vs 9.6 [4.4] nmol/l; p=0.0065) but similar free 25OHD (16.7 [10.4] vs 17.8 [7.5] pmol/l p=0.6530). After dosing, total 25OHD rose similarly in each group (\approx 56 nmol/l), but measured free 25OHD rose more in Asians (18.1 [9.4] vs 12.2 [13.3] pmol/L p=0.0464). Lower DBP at baseline, possibly reflecting genotype differences, was associated with a greater change in measured free 25OHD in Caucasians, but not in Asians.

Conclusions

Asian compared with Caucasian males had a larger increment in measured free 25OHD following 150,000 units vitamin D₃, possibly reflecting differences in DBP affinity for 25OHD. Ethnicity should be considered when devising guidelines for the treatment of vitamin D deficiency.

Key words:

Bio-available 25 hydroxy vitamin D, Ethnicity, Serum Measured Free 25 hydroxy vitamin D, Vitamin D binding protein, Vitamin D binding protein genotype, Vitamin D₃.

MAIN TEXT FILE

Introduction:

Vitamin D deficiency is a term widely used but rarely defined in terms of functional outcomes. Based on synthesised evidence, thresholds for low dose supplementation or higher dose treatment have been suggested. The Scientific Advisory Committee on Nutrition Department (SACN) recommends a threshold of 25 nmol/L for serum 25OHD for all individuals at any time of the year to protect the musculoskeletal health.[1] The Institute of Medicine defines the threshold for vitamin D deficiency as ≤ 30 nmol/L.[2] The Endocrine Society defines the threshold for vitamin D deficiency as < 50 nmol/L, which would likely include more than half the population of the UK during winter months.[3]

What remains unclear is the extent to which “one size fits all” in providing vitamin D supplementation or treatment. In the UK, vitamin D deficiency is widely reported, more so in those with darker skin including those of African or Asian descent. The effect of ethnicity, independently of other factors, has not previously been considered when devising strategies and recommendations for either low dose supplementation or higher dose treatment.

Vitamin D status is currently assessed by measuring circulating 25-hydroxyvitamin D (25OHD), which exists either free in the circulation ($< 1\%$), or bound to albumin or the vitamin D-binding protein (DBP). The terms “free” vitamin D and “bioavailable” vitamin D refer respectively to unbound 25OHD, or unbound plus 25OHD bound to albumin. The extent to which the total measurement adequately reflects either free or bioavailable serum 25OHD and whether any relationship between “total” and “free” varies following supplementation, or with other factors such as ethnicity, is unclear. Reports of higher free and bioavailable serum 25OHD for the same total serum 25OHD in African Americans have been challenged because of concerns regarding DBP measurement accuracy [12,13,15,16,17].

In light of this, we sought to investigate whether there were differences between serum total and directly measured free 25OHD concentrations between Asians and white Caucasians and the changes in these parameters following administration of a single dose of 150,000 IU of vitamin D₃. In addition, we aimed to determine the effect of covariates including DBP concentration and genotype, concurrent diet and skin colour on both the free and total 25OHD responses, and the extent of parathyroid hormone (PTH) suppression following dosing.

Materials and methods:

Study design: This was an exploratory study to determine the size of the effect on serum free and total 25OHD of a 150,000 unit dose of vitamin D₃, given to young adults from different ethnic groups (Figure 1).

Study participants: We recruited a cohort of sixty apparently healthy young adult men from two ethnic groups - White Caucasian (n=30) or South/East Asian (n=30) origin. Eligibility criteria included men aged between 18 and 25 years who were free from any condition affecting bone health, general nutrition, growth and glucose metabolism. Subjects with any chronic illness involving the liver and kidney, causing malabsorption, who used steroids, anticonvulsants, or vitamin D treatment as well supplementation or any medication that might affect calcium and vitamin D metabolism, were excluded. Recruitment took place within the University of Sheffield using a mixture of email, posters/leaflets and pre-lecture 2-minute talks. Subjects were students of the University of Sheffield, mostly medical and dental students. None of the study participants travelled to destinations where cutaneous vitamin D synthesis could have taken place during the study period. Whilst it was anticipated that gender should not impact on the outcome, the administration of a large dose of vitamin D₃ might have had unexpected effects on an early stage pregnancy, thus we excluded young women.

Demography and auxology: At baseline, height (without shoes to next succeeding 1mm by wall-mounted stadiometer [Holtain, Crymych]), weight (wearing vest and pants to nearest 0.1 kg by Marsden portable weighing scales, body mass index [BMI (kg/m²), and waist:hip circumference ratio (paper tape measure) were recorded in all subjects.

Dietary calcium and vitamin D intake: A validated 101-item food frequency questionnaire (DIET-Q) was used to ascertain the dietary intake of calcium and vitamin D at baseline. Mean daily calcium (grams/day) and vitamin D (IU/day) intake were analysed using Q Builder (V4.0) nutritional software (Tinuviel software, Llanfechell, Anglesey UK).

Skin type, season and sun exposure: Skin type was assessed using a 6-point Fitzpatrick scale in all subjects at baseline [4]. Fitzpatrick 1 skin type is fair and freckled; type 6 is very dark/black. The study was conducted in the UK during January and February to avoid sun exposure.

Samples:

Baseline: Fasting blood samples were collected for serum total 25OHD, free 25OHD, calcium, phosphate, magnesium, albumin, alkaline phosphatase, PTH, DBP and DBP genotype.

Four weeks: All blood investigations (except DBP genotype) were repeated.

Urinary calcium:creatinine ratio: A fasting second void urine sample for calcium:creatinine ratio was collected at baseline, 1 and 4 weeks after vitamin D3 administration.

Vitamin D3 dosing:

A single dose of 150,000 IU of Vitamin D3 [6 mls of Invita D3 (Consiliant) 25,000 IU/ml oral solution] was administered under direct supervision. We chose this dose based on the work done by Oliveri B et al where the authors demonstrated the safety of a single dose of 150,000 IU of vitamin D to maintain appropriate levels of 25OHD without causing hypercalcaemia or hypercalciuria [5].

Laboratory methods:

Serum total 25OHD: Serum total 25OHD levels were determined using an UPLC/Mass Spectrometer Semi-automated hexane extraction in the Acquity Ultra Performance LC/Quattro MS (Waters) analyser. Lower limit of detection for 25OHD₂ was 6 nmol/L and for 25OHD₃ 3.5 nmol/L. The interassay coefficient of variation (CV) for 25OHD₂ and 25OHD₃ were 5.7% and 5.4% respectively.

Serum free 25OHD: Free 25OHD levels were measured using an ELISA from Future Diagnostics Solutions. The interassay CV was 4.8%.

Serum Vitamin D binding Protein: DBP levels were measured using an ELISA from Genways Biotech Inc. The interassay CV was 5.8%.

Serum calcium, phosphate, albumin, and alkaline phosphatase; urine calcium, creatinine: Measured using Micro Slide Technology Colorimetric/Rate by Reflectance Spectrophotometry in the Vitros 5, 1 FS System (Ortho Clinical Diagnostics) analyser. The interassay CVs were: calcium (1.4%), phosphate (1.6%), albumin (2.9%), alkaline phosphatase (2.4%), urine calcium (1.7%) and urine creatinine (4.4%).

Intact Parathyroid hormone (PTH): PTH was measured using Immunoassay (Chemiluminescent Microparticle Immunoassay) in the Architect *i* 1000 System (Abbot) [PTH analytical sensitivity ≤ 1 ng/L].

DBP genotyping: A pyrosequencing assay was developed in house, using PSQ assay design software version 1.0.6 (Qiagen), to detect two single-nucleotide polymorphisms (SNPs), rs4588 and rs7041 in the DBP gene, that give rise to 3 common variants of DBP (i) Gc1f (ii) Gc1s and (iii) Gc2. All subjects were genotyped for 6 different haplotypes - Gc1f-1f, Gc1f-1s, Gc1f-2, Gc1s-1s, Gc1s-2, and Gc2-2, ranked here in order of 25OHD binding affinity, highest to lowest.

PCR and sequencing primers were as follows:

F: 5' -ATCTGAAATGGCTATTATTTTG-3',

R: 5' Btn -ACAGTAAAGAGGAGGTGAGTT-3',

Seq: 5' -AAAAGCTAAATTGCCTG-3'.

To ensure distinct pyrosequencing signals, (~10 ng) of human genomic DNA was amplified by 47× PCR cycles using OneTaq® 2X Master Mix with Standard Buffer (NEB). For each genotype determination, single-stranded DNA was purified from 5 µl of PCR products using PyroMark Q96 Vacuum Prep Workstation (Qiagen). PCR products were denatured to single-stranded DNA (ssDNA) and annealed with sequencing primers. Pyrosequencing was then performed on a PyroMark Q96 MD Instrument according to manufacturer's instructions (Qiagen). Nucleotide dispensation order was as follows: DBP rs4588+rs7041 CATGTCACACTG. SNP analysis was carried out using the SNP analysis software provided (Qiagen).

Calculated free and bio-available 25OHD: Free and bioavailable 25OHD levels based on fixed affinity constant for the DBP genotype (Gc1f-1f) was calculated using the mathematical model described by Chun et al [6].

Sample size:

This was an exploratory study to determine effect size and variance; hence no formal calculation of sample size was undertaken. We sought advice from the Yorkshire and Humber Research Design Service who suggested that a sample size of 30 per group was sufficient to undertake exploratory work of this nature.

Statistical methods:

Continuously distributed data was summarized by the median (25th/75th centiles); categorical data by n(%).

Missing values are tabulated but not considered otherwise in our analysis.

Normality checks were carried out; data were generally normally or near-Normally distributed, hence parametric tests were used to assess differences between groups.

To compare the groups prior to dosing, 2 sample t-tests of baseline characteristics were performed and are reported in Table 1.

The main question of interest was whether ethnicity impacted on change in total and free 25OHD following dosing. Two sample t tests were carried out to compare the changes in total and free 25OHD between the groups. Analysis of variance was also used to determine any other statistical differences between the groups and adjust for covariates. Generalized linear models were used to determine statistical differences between response variables that had error distribution models other than normal, and to assess the interactive effect of ethnicity and DBP on change in free 25OHD.

Fisher's exact test was used to compare the categorical data. Graphical presentation was made by Box and Whisker plot. P values were used sparingly with an arbitrary threshold of 0.05(two tailed).

We performed all our analyses using Statistical Package for the Social Sciences version 22 (SPSS by IBM), Data Desk™ v6.2.1, and Stata v14 [7]. For method comparison between directly measured serum free 25OHD and calculated free 25OHD, we used Medcalc for Windows, version 8.0 (Medcalc Software, Mariekerke, Belgium) to perform Bland-Altman analysis[8] and Passing Bablok regression[9].

Results:

Baseline clinical characteristics

The baseline clinical characteristics of the study subjects are shown in Table 1. The white Caucasian and Asian men did not differ with respect to age, weight, height, BMI and waist to hip ratio. The mean (SD) Calcium intake (grams/day) was adequate and equal between the Asians [1031.23(382.6)] and white Caucasians [1065.87(266.88)]. The median (range) vitamin D intake (IU/day) in comparison with the UK recommended daily intake was low and similar between the groups, 140(74,276) and 120(20,524) in Asians and white Caucasians respectively. White Caucasians had a Fitzpatrick skin type of 1-3 and Asians 3-5. White Caucasians predominantly had Gc1s-2 haplotype and did not have Gc1f-1f and Gc1f-2. South Asians predominantly had Gc1s-1s haplotype and East Asians Gc1f-1f haplotype. Gc2-2 and Gc1s-2 were not found in East Asians (table 2). Table 2 shows the relationship between haplotype frequency and ethnicity. There was a significant association between the two ($p=0.052$, fisher's exact test). There were more Asians with Gc1f-1f haplotype than expected [5 vs 2.5]. Similarly, more Caucasians with Gc2-1s haplotype than expected [11 vs.8.5].

Table 2 shows the differences in baseline total 25OHD status between the different haplotypes and ethnicity. However, the numbers were too small for statistical comparison.

Influence of ethnicity on measured parameters at baseline and following intervention

Twenty-nine participants out of the 60 recruited had a serum total 25OHD level of < 30nmol/L at baseline, of which 60% (18/30) were Asian and 36% (11/30) were white Caucasian (Table1). Asians had significantly lower serum total 25OHD and DBP levels, but similar measured and calculated free 25OHD levels compared to white Caucasians at baseline (Table 3). At four weeks the changes in serum total 25OHD, DBP, and calculated free 25OHD levels were similar between Asians and white Caucasians; however the increase in directly measured serum free 25OHD level was significantly greater in Asians 18.1(9.4) vs 12.2 (13.3) pmol/L in Caucasians ($p=0.0464$) (Table 3) (Figure(s) 2a, 2b and 2c). Although the observed significance was marginal, we considered it to be acceptable for a preliminary study.

There was a significant interaction ($p<0.01$) between ethnicity and mean DBP in relation to change in free 25OHD following dosing. In Caucasians, a lower mean DBP was associated with a larger increase in measured free 25OHD; this was not true for Asians, in whom the relationship of free 25OHD with mean DBP was positive, i.e as mean DBP increased, the change in free 25OHD also increased (Figure 2d).

We found no clear effect of DBP haplotype within ethnic group on change in either total or free 25OHD, either alone or in combination with other factors.

Baseline PTH concentration was higher in Asians than in white Caucasians ($p=0.0019$) (Table 3). Following intervention, no significant changes in PTH levels were noted in either of the groups (Table 3). No increase in urine calcium: creatinine ratio was noted at either 1 or 4 weeks post intervention.

Method comparison between directly measured and calculated serum free 25OHD concentration using fixed affinity constant for the DBP genotype (Gc1f-1f)

Bland-Altman plot at baseline shows clear evidence of bias and one outlying observation (Figure 3a). Passing

Bablok plot at baseline shows small and non significant systematic bias (intercept = 0.54, 95% CI -3.54, 3.05 pmol/L). There is a significant proportional bias (slope = 0.43, 95% CI 0.24, 0.71 pmol/L) (Figure 3c).

Bland-Altman plot post supplementation shows no evidence of bias (Figure 3b).

Passing Bablok plot post supplementation shows a large negative systematic bias (-13.29 pmol/L). This is not statistically significant (95% CI -33.82, 2.99 pmol/L). There is no significant proportional bias (slope =1.04, 95% CI 0.54, 1.70 pmol/L) (figure 3d).

Relationship of PTH with total and free 25OHD

There was a statistically significant negative correlation between PTH and total 25OHD at baseline ($r=-0.442$; $p=0.0006$) and post intervention ($r=-0.452$; $p=0.0004$). However, the relationship of PTH at baseline with measured free 25OHD did not reach significance at baseline ($r=-0.245$; $p=0.0634$) or following intervention ($r=-0.061$; $p=0.6446$).

Relationship of skin type with total and free 25OHD

There was no statistically significant difference between skin types and baseline total 25OHD concentrations by one-way ANOVA ($F(4,54)=2.08$, $p=0.0956$). Post-dosing total 25OHD also did not differ between skin types.

Relationship of DBP genotypes on total and free 25OHD

There were no significant differences by DBP genotype for baseline total 25OHD (F ratio =1.0075, $p=0.4225$) or serum free 25OHD (F ratio =0.4838, $p=0.7868$). Following intervention, subjects with Gc1f-1s haplotype (high affinity for 25OHD) showed the greatest increment in serum total 25OHD (increment 60.65 (17.3) nmol/L;

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baseline 32.9 (14.0) nmol/L). Subjects with the lowest affinity haplotype Gc2-2 had the smallest increment in serum total 25OHD (increment 48.9 (6.9) nmol/L, baseline 18.4 (3.6) nmol/L). The increment in serum direct free 25OHD levels was greatest in subjects with the Gc2-1s (16.6 (10.2) pmol/L; low affinity haplotype), and lowest in subjects with Gc1f-1f (10.1 (11.9) pmol/L highest affinity haplotype). None of these differences reached statistical significance, however.

Relationship of dietary calcium and vitamin D intake with PTH, DBP, total and free 25OHD

There was no relationship of dietary calcium intake at baseline with baseline PTH, DBP, total or free 25OHD, or change in any of those parameters, either for the whole group or by ethnicity.

Discussion:

We found that serum total 25OHD was low in young men of White Caucasian and Asian ethnic origin during winter in Sheffield and increased similarly in both groups following administration of 150,000 units of vitamin D₃, but that measured free 25OHD increased more in those of Asian origin. We found a relationship between both baseline and mean DBP concentration and incremental change in measured free 25OHD according to ethnicity. As mean DBP rose, the incremental increase in free 25OHD reduced in Caucasians, and increased in Asians. This suggests that the binding affinity of DBP may vary with ethnic origin.

There is a biochemical basis for the differences in affinity of the different DBPs for vitamin D metabolites.

Polymorphisms in the Gc gene (codominant alleles) give rise to three major electrophoretic variants of Gc (Gc1f, Gc1s, and Gc2), which differ by amino acid substitutions as well as glycosylation[9]. The amino acid sequence of the three isoforms 1f, 1s and 2 differ at positions 152, 311, 416 and 420[11]. The position 416 difference is functionally important as this site contains a branched trisaccharide that in turn is linked to sialic acid (1f), mannose (1s) or no sugar residue (2). Such post-translational modifications are likely to affect the properties of the isoforms. Indeed, the relative affinity constants differ, so that if 1f is assigned a value of 1, then the values for 1s and 2 are 0.536 and 0.321, respectively [12,6].

Calculated estimates of free 25OHD were found to be significantly different from direct measurements, and showed a systematic bias. According to the free hormone hypothesis [13], the biological action of 25OHD is exerted by its freely-available form (<1% of the total), not by the total circulating amount which comprises DBP-bound (85-90%) and albumin-bound (10-15%) forms plus free. There is support also for the concept of bioavailable 25OHD[14], comprising the albumin-bound and free fractions, suggesting that measurement of free or bio-available 25OHD concentration may provide a more meaningful marker of vitamin D function than total [15].

The movement of DBP-bound 25OHD to bioavailable or free forms likely depends in part on the concentration of DBP and its binding affinity for 25OHD. Both are known to vary significantly both by and within ethnic groups. A recent report by Yao et al has also demonstrated significantly lower levels of DBP ($p < 0.001$) measured using a monoclonal assay (165.3 \pm 90.4 ug/ml) in comparison with the polyclonal assay (418.7 \pm 99.0 ug/ml) in a Chinese population [16]. Powe et al in their cross sectional study reported that black Americans have lower total 25OHD and DBP resulting in similar concentrations of calculated bio-available 25OHD compared to white counterparts [14]. Bouillon et al showed similar results in Black Gambians compared to White Caucasians using the same DBP assay. In contrast, when they measured DBP using a polyclonal assay (as used here), in the same cohort, they did not find any difference between the groups [17]. Similarly, Aloia et al. reported identical concentrations of DBP between US blacks and whites, using a polyclonal assay [18]. Recently, Nielson et al. compared the DBP assays used in the MrOS [19] and MRC cohorts [20] and characterised the molecular forms of DBP [21]. The authors reported that the difference in DBP levels between the Africans and Whites identified using a monoclonal assay disappeared when measured using polyclonal or proteomic methods [21]. This contrasts with our results; we found a significant difference in DBP levels between the Asians and White Caucasians using the polyclonal assay. Lower DBP levels have previously been documented in carriers of two Gc2 alleles [22-24]. We found no clear effect of genotype either at baseline or following intervention on serum DBP, irrespective of ethnicity. Since our population size is small the results in relation to DBP and genotype should be considered preliminary and thus need confirming in a larger population.

We found directly measured serum free 25OHD at baseline in Asians and Caucasians to be very similar despite lower total 25OHD in Asians. We hypothesized originally that the lower DBP concentrations found in the Asians in our cohort were likely to be the reason for their comparable levels of free 25OHD. Following intervention with vitamin D3, no change in DBP levels were observed in either Asians or White Caucasians when compared to baseline, indicating that the serum DBP concentration is not altered by single dose supplementation. Our findings agree with those of with Sollid, where no effect of vitamin D3 on serum DBP concentration was shown in an interventional trial (20,000 IU D3 weekly for a year) in Caucasians [24].

We found a higher increment in measured free 25OHD concentration in Asians following vitamin D3 supplementation, despite a similar increment in total 25OHD concentration and no significant change in DBP concentration. In addition, the incremental increase in Caucasians' serum free 25OHD was inversely related to both baseline and mean DBP, whereas in Asians it was not. This implies that DBP affinity for vitamin D and its metabolites may be a key factor in the response of Asians to vitamin D treatment. The lack of a clear relationship with DBP genotype could imply that additional factors may be at play, or may be due to the small sample size.

Alzaman et al. compared the differences in total and free 25OHD levels between Black and White Americans following daily D3 supplementation (2000 IU or 4000 IU) or placebo for a total of sixteen weeks in nearly 200 diabetic subjects. The authors found similar and dose-proportionate increases in both total and free 25OHD in both groups [25]. Sollid et al. studied the relationships between serum total and free 25OHD (both directly and by calculation) in relation to age, sex, BMI, season and DBP genotype and their inter-relationship with the weekly administration of 20,000 units of D3 / placebo for a year in nearly 500 individuals. The authors found that serum DBP was not affected by vitamin D supplementation. They demonstrated that age, sex and DBP concentration did not affect increment in vitamin D parameters, following supplementation [24].

If serum free 25OHD increases disproportionately in some ethnic groups following vitamin D administration, is this a problem? There is another step beyond 25-hydroxylation in producing the biologically active form of vitamin D (1,25(OH)₂D), and this step is highly regulated to protect against hypercalcaemia. The finding here that PTH did not decrease as expected in White Caucasians as both free and total serum 25OHD increased is puzzling. However, PTH did decrease in the Asian group; this suggests that the greater increase in free 25OHD in Asians may have broader biological significance in terms of calcium metabolism, perhaps reflecting increased calcium absorption. Asians had lower baseline total serum 25OHD, and are more likely (as a population group) to receive treatment. The concern would be therefore that significantly increasing calcium absorption might have unexpected and undesirable consequences in an ethnic group already at higher risk for cardiovascular and renal disease.

In the method comparison between the calculated and directly measured serum free 25OHD using fixed affinity constant for the DBP genotype, there was a positive but not significant systematic bias at baseline. Post supplementation the systematic bias was negative and also non-significant. We cannot explain this finding. This may be due to small sample size (figure 3a,b,c,d).

Strengths and Limitations:

Ours is the first interventional study reported so far, studying the effects of single dose of vitamin D₃ on serum total 25OHD, DBP and free 25OHD concentration in both Asians and Caucasians. The main limitation of our study is the small sample size; hence we had an insufficient statistical power to demonstrate the influence of skin types or DBP genotypes on serum 25OHD (total and free) with or without vitamin D₃ supplementation. We conducted the study exclusively in young healthy males; hence our study findings may not be applicable to conditions associated with variation in DBP levels such as gender, pregnancy, liver disease and infections, and may not necessarily be applicable across all ages. We also included no functional outcome measures.

Conclusion:

Our primary aim was to determine the size of the effect of vitamin D supplementation in different ethnic groups. In an era of “precision medicine” we should be able to better target vitamin D treatment. Rather than adopting a “one size fits all” policy, we should aim to develop measurements that accurately reflect vitamin D status across all ethnic groups, and use these to guide treatment for relevant functional outcomes.

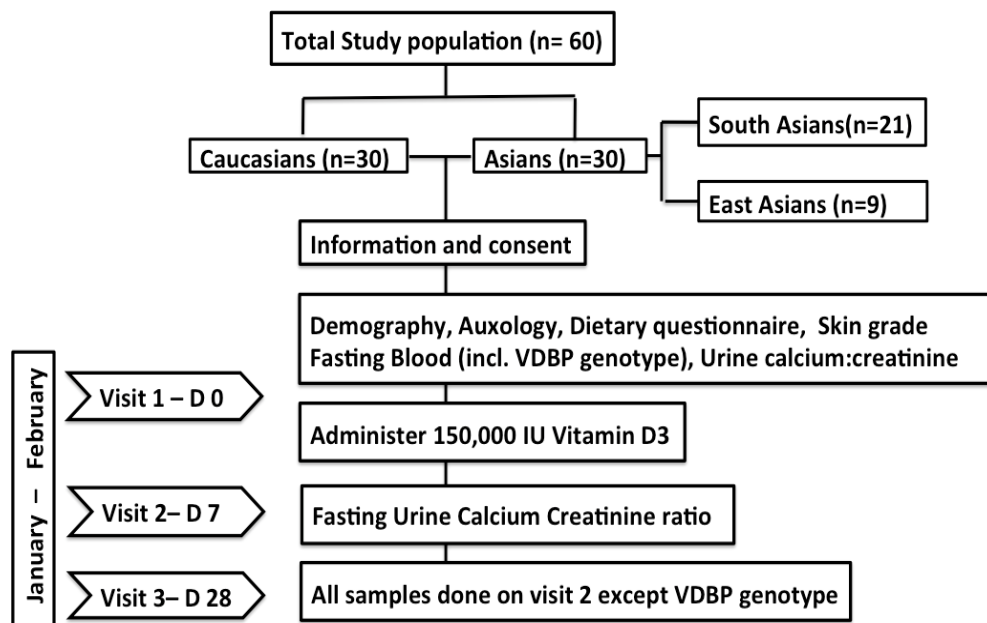
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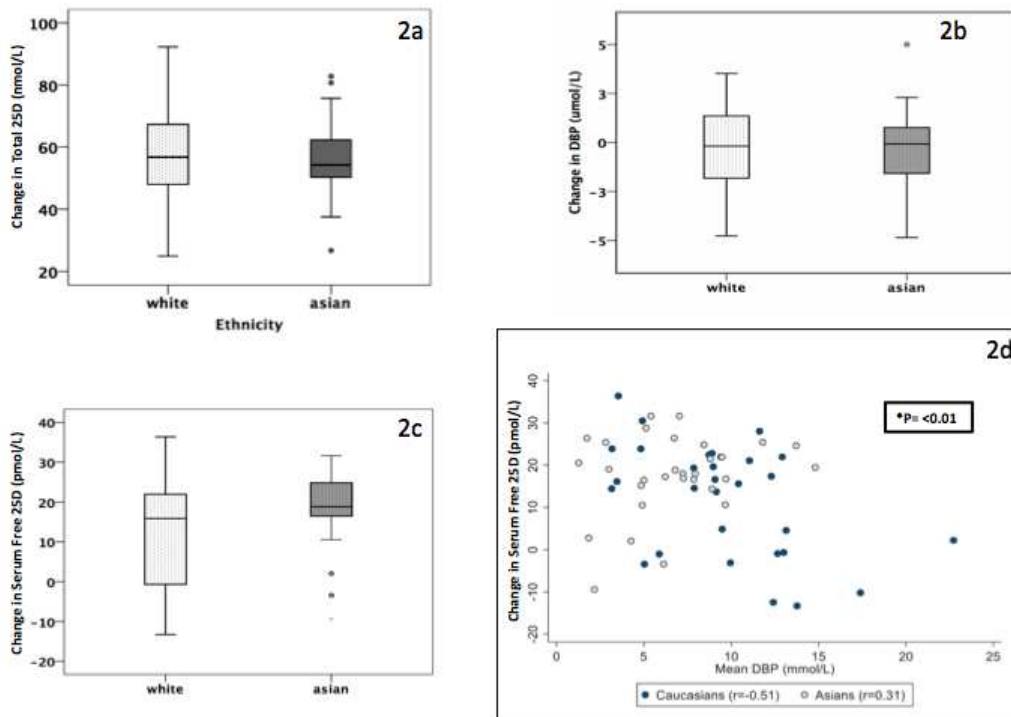
Title: Figure 1. Study design



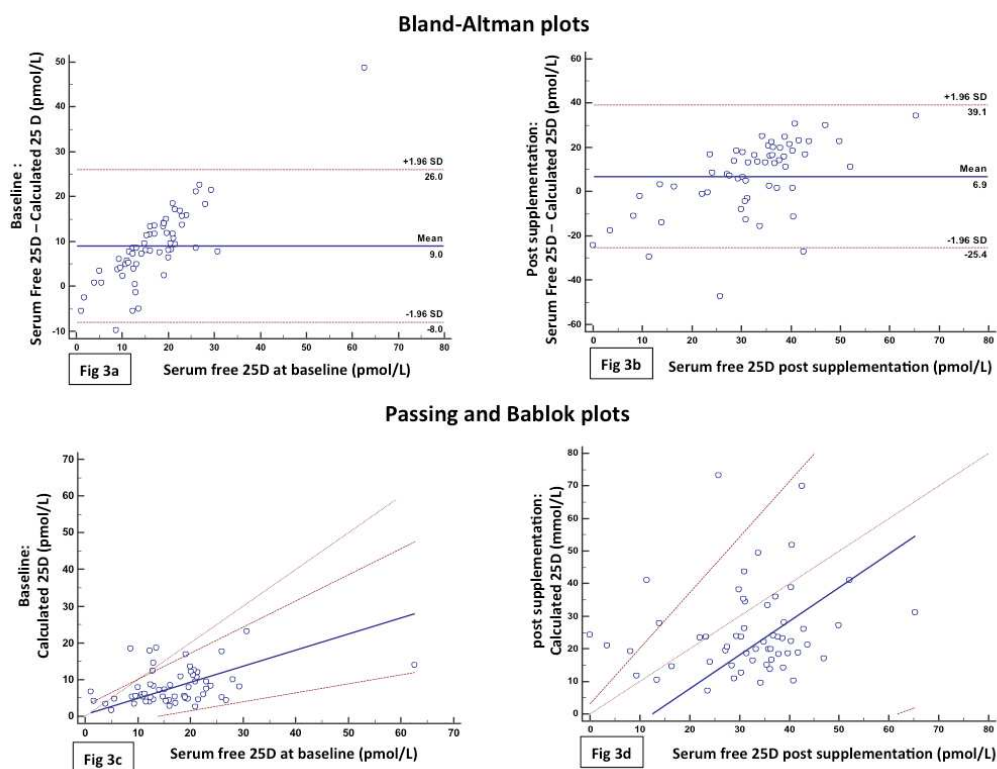
Legend: Figure 1 showing the design of the study

Title: Figure(s) 2a,b,c: Change in Total, Free 25OHD and DBP following vitamin D dosing.
Figure 2d: Change in serum free 25OHD according to mean DBP

Legend: Figure(s) 2a,2b,2c show the change in total, free 25OHD and VDBP following vitamin D dosing. Three-part box plot showing ethnicity in X-axis and change in Total 25OHD, VDBP and Free 25OHD in Y-axis. Figure 2d shows the change in serum free 25OHD according to mean DBP with shaded circles representing Caucasians and clear circles Asians.



Title: Figure (s) 3a.b.c.d. Method comparison: Direct vs. Calculated Free 25OHD-post supplementation (using fixed affinity constants)



Legend:

Figure 3a and 3b show the Bland-Altman plots. Bold line indicates mean difference with 95% limits of agreement on either side. Figure 3a demonstrates systematic bias between calculated (using fixed affinity constants) and directly measured serum free 25OHD at baseline and figure 3b, which is post supplementation, does not. Mean serum free 25OHD (directly measured) is represented in X axis and the difference between the directly measured and calculated free 25OHD is represented on the Y-axis. Bland-Altman plots the mean of X and Y versus the difference between X and Y.

Figure(s) 3c and 3d show the Passing and Bablok plots. Bold line indicates the calculated regression. With 95% CI either side. The line of no difference is drawn at 45 degrees.

Figure 3c demonstrates no systematic bias but a significant proportional bias at baseline. Figure 3d demonstrates neither systematic nor proportional bias post supplementation. Serum free 25OHD (directly measured) is represented in X axis and the calculated free 25OHD (using fixed affinity constants) represented on the Y-axis. Passing and Bablok is a nonparametric regression for method comparison. It fits a straight line between two variables (X and Y) where both are measured with error. The intercept of the straight line represents the systematic bias between X and Y. The null hypothesis is zero intercept. If the 95% confidence (CI) crosses zero the systematic bias is not statistically significant. The slope measures the amount of proportional bias. The null hypothesis is a slope of one. If the 95% CI cuts across on the proportional bias is not statistically significant. If the two methods are identical, they same they will share a common intercept (zero) and a common slope (one).

Title: Table 1. Baseline demographics table, mean (SD)

	Whites	Asians	P value
Study population	30	30	-
Age (years)	21.43 (1.54)	22.33 (1.44)	0.66
Weight (kg)	76.97 (9.16)	70.77 (8.34)	0.34
Height (cms)	179.95 (5.59)	175.77(5.6)	0.95
BMI	23.76 (2.51)	22.89 (2.30)	0.61
Waist : Hip ratio	0.81 (0.05)	0.88 (0.09)	0.30
Calcium intake (gms/day)	1065.87 (266.88)	1031.23 (382.6)	0.28
Vitamin D intake (iu/day)*	120 (20, 524)	140 (74,276)	0.29
Fitzpatrick skin grade	1 - 3	3 - 5	-
Serum Calcium (mmol/L)	2.31 (0.06)	2.30 (0.05)	0.49
Serum Phosphate (mmol/L)	1.38 (0.19)	1.36 (0.15)	0.74
Albumin (g/L)	44.2 (2.2)	43.7 (1.7)	0.32

Legend: Table 1 showing the baseline demographic data and the values are expressed in mean (SD) with the p values.

Title: Table 2. Haplotype frequencies by ethnicity and median 25OHD concentration at baseline

Haplotype		Caucasian	Asian	Total	Haplotype	Caucasian	Asian
						Median Baseline 25OHD concentration (nmol/L)	
Gc1f-1f	Obs	0	5	5	Gc1f-1f	-	-
	Exp	2.5	2.5				
Gc1s-1f	Obs	9	6	15	Gc1s-1f	31.6	20.4
	Exp	7.5	7.5				
Gc1s-1s	Obs	9	8	17	Gc1s-1s	40.8	20.4
	Exp	8.5	8.5				
Gc2-1f	Obs	0	3	3	Gc2-1f	-	-
	Exp	1.5	1.5				
Gc2-1s	Obs	11	6	17	Gc2-1s	33.2	24
	Exp	8.5	8.5				
Gc2-2	Obs	1	2	3	Gc2-2	22.4	16.4
	Exp	1.5	1.5				
Total		30	30	60	Fisher's exact test, p=0.052		

Legend: Table 2 showing the haplotype frequencies by ethnicity as well the difference in median 25OHD concentration (nmol/L) between Caucasians and Asians based on their haplotypes.

Title: Table 3. Results showing serum total and free 25OHD levels, estimated 25OHD and PTH levels at baseline and increment post supplementation, mean and SD

		Serum Total 25OHD (nmol/L)	Serum DBP (umol/L)	Measured Free 25OHD (pmol/L)	Calculated Free 25OHD(pmol/L)	PTH (ng/L)
Baseline	Caucasians	34.1 (12.3)	6.6 (3.0)	17.8 (7.5)	13.6 (7.8)	44.6 (14.2)
	Asians	26.3 (13.7)	4.7 (2.3)	16.7 (10.4)	11.9 (6.8)	69.8 (38.6)
*p-value <0.05	p value	*0.004	*0.001	0.65	0.38	*0.0019
Increment	Caucasians	56.7 (18.3)	0.31 (2.0)	12.2 (13.3)	24.4 (14.5)	2.2 (14.2)
	Asians	56.2 (12.6)	0.24 (2.0)	18.1 (9.4)	29.4 (20.1)	- 4.7 (27.7)
*p-value <0.05	p value	0.90	0.90	*0.0464	0.29	0.24

Legend: Table 3 showing serum total and free 25OHD levels [both measured and calculated], vitamin D binding protein (DBP) and parathyroid hormone (PTH) levels at baseline and their increment post supplementation, expressed in mean (SD).